PATENT
Docket No.: I9000.0058/P058

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Marit Nilsen-Hamilton

Serial No.: 10/809,886

Group Art Unit: 1635

Filed: March 26, 2004

Examiner: Kimberly Chong

For: ALLOSTERIC PROBES AND METHODS

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF MARIT NILSEN-HAMILTON UNDER 37 CFR 1.131

Dear Sir:

I, Marit Nilsen-Hamilton, declare and state as follows:

1. I am of legal age, and under no disability that prevents me from attesting to the following statements and information, which are based on my personal knowledge and observations or on my best information and belief.

2. I reside at 1111 N. Hyland Avenue, Ames, Iowa 50014.

3. I am the sole inventor of the above-identified U.S. patent application (the "'886 application"), filed on March 26, 2004, as evidenced by the attached executed Declaration (Exhibit A).

4. I reviewed and understand the above-identified U.S. patent application including the currently pending claims and amendments (the "Claimed Invention").

5. I conceived of and reduced to practice the invention covered by the Claimed Invention prior to April 24, 2002. My reduction to practice of the Claimed Invention is evidenced by Exhibits B-D, which are summaries documenting work I directed and supervised. Specifically, Exhibits B-D are photocopies of handwritten notes, and electronic summaries of the handwritten notes, both created by my student who conducted experiments under my direction and supervision. Each of the experiments summarized in Exhibits B-D was conducted by my student under my direction and supervision prior to April 24, 2002, although the electronic summaries were printed after April 24, 2002. The actual dates that the experiments were conducted have been blanked out, as has any description not relevant to the conception of the Claimed Invention; however, the dates are all prior to April 24, 2002.

6. Exhibits B-D shows the results of an experiments involving CLAMP 609, which is a probe for binding a plurality of targets comprising an allosteric regulator linked to at least one regulated aptamer. Specifically, as noted in Exhibit D, CLAMP 609 includes an allosteric regulator, which is a nucleic acid molecule, linked to a regulated aptamer in cis configuration.

7. The experimental results described in Exhibits B-D show that binding the allosteric regulator with a first target enhances the binding of the at least one regulated aptamer to at least one second target. Specifically, as shown in Exhibits B-D, when neomycin binds the allosteric regulator of the CLAMP 609, the regulated aptamer is increasingly able to bind ATP.

8. Thus, Exhibits B-D show that, prior to April 24, 2002, the Claimed Invention was reduced to practice under my direction and supervision.

All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true. All statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent.

Date: 9/27/06

By: Marit Nilsen-Hamilton

Marit Nilsen-Hamilton

EXHIBIT A

Declaration for Patent Application English Language Declaration	Attorney Docket No.	I9000.0058/P058
	First Named Inventor	Marit Nilsen-Hamilton
	COMPLETE IF KNOWN:	
☐ Submitted with initial filing ☒ Submitted after initial filing (surcharge required 37 CFR 1.16(e))	Application No.	10/809,886-Conf. #7776
	Filing Date	March 26, 2004
	Art Unit	1645
	Examiner	Not Yet Assigned

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ALLOSTERIC PROBES AND METHODS

the specification of which

☐ is attached hereto

OR

☒ was filed on 03/26/2004

as United States Application No. or PCT International Application No. 10/809,886

and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign applications(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's right certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

			Priority Not Claimed	Certified Copy Attached	
				YES	NO
_____ (Number)	_____ (Country)	_____ (Filing Date)	☐	☐	☐
_____ (Number)	_____ (Country)	_____ (Filing Date)	☐	☐	☐
_____ (Number)	_____ (Country)	_____ (Filing Date)	☐	☐	☐

☐ Additional prior foreign applications are listed on a supplemental data sheet attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Marit Nilsen-Hamilton	
Sole or first inventor's signature Marit Nilsen Hamelta	Date 9/2/04
Residence Ames, Iowa	
Citizenship Norway	
Mailing Address 1111 N. Hyland Ave. Ames, Iowa 50014-4007	

STATE OF IOWA)
) ss:
COUNTY OF STORY)

On this 2nd day of September, 2004, before me personally came Marit Nilsen-Hamilton, to me known to be the individual described in and who executed the foregoing instrument, and acknowledged execution of the same.

EXHIBIT B

TITLE: Column Affinity - CLAMP 609 on ATP Agarose in Neomycin Solution | DATE:
PURPOSE: To test the effect of free neomycin on Neo/ATP-CLAMP 609 binding | EXP.# BK036

REAGENTS

DEPC treated water
Neo/ATP-CLAMP 609 (~ 0.5pmol/ul) BK035 (gcuuaauacgacucacuauaggccugggcgagaaguuuaggccuu ggguugggaagaaacuguggcacuucggugccagcaaccc)
Neomycin Sepharose from BK001
ATP Agarose from Sigma No. A2767
MicroBioSpin Column from BioRad No. 732-6204
Neomycin from Sigma No. N-1876 Lot 90K0854 MW 908.9
Adenosine 5'Triphosphate (ATP) from Sigma No. A-3377 MW 551.1
Binding Buffer: 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6
Binding Buffer (ATP columns): 1mM neomycin in Binding Buffer above
Elution Buffers: 10mM Neomycin or 10 mM ATP in Binding Buffer
Fisher Scientific ScintiVerse Scintanalyzed Cocktail No. SX18-4
Packard TR1600 Liquid Scintillation Analyzer (1238 Molec Biol) - Protocol 3

STEP	SOLUTION	VOL (ml)	TEMP (°C)	Time Period (I
Make Column	Add 100ul either neomycin sepharose or ATP agarose to each column.	100ul	23	5min
Wash Column**	Equilibrate column by washing 10x with 100ul Binding Buffer, collecting 1ml.	1x1ml/fx	23	5min
Prepare sample	Add ~ 1pmol ^{32}P-CLAMP609 to DEPC water for each sample and preincubate.	20ul	85 23	1min 1min
Apply Sample	Apply sample to column.	18ul	23	30sec
Wash	Wash column 20x with 100ul Binding Buffer, collecting each 1ml.	2x1ml/fx	23	10min
Elution	Elute 10x with 100ul Elution Buffer, collecting each 100ul.	10x100ul/fx	23	10min
**Column Wash	Wash column 10X with 100ul Elution Buffer, then 10x with 100ul Binding Buffer, collecting each 1ml.	2x1ml/fx	23	10min
Analyze Fractions	Add 20ul each fraction to 5ml ScintiVerse and assay on liquid scintillator. Reserve 2ul of sample for total sample cpm count.	20ul in 5ml	23	112min

**Column Washes only for ATP agarose column

DISCUSSION:

The neomycin in the binding buffer apparently increased the effective ATP binding of CLAMP 609. In th first trial, 90% of the TCA precipitable counts bound to the neomycin sepharose and 17% were eluted from the ATP agarose (with only about 50% of the total radioactivity recovered from the column).** In th second trial, again about 90% bound to the neomycin sepharose and 35% bound to the ATP agarose, with much less radioactivity remaining in the column (about 80% recovered). This is a great improvemer over the inital CLAMP 609 binding assays (BK033 & BK034) where less than 10% was shown to bind to the ATP agarose. Next this difference in binding must be directly compared in a single experiment with and without neomycin in the binding buffer.

**Note, the elution in the first trial with ATP was distributed over the entire elution series and was probat background. However, in the second trial there was a definite peak of CLAMP binding

INIT____DATE:____PRINTED: May 13, 200

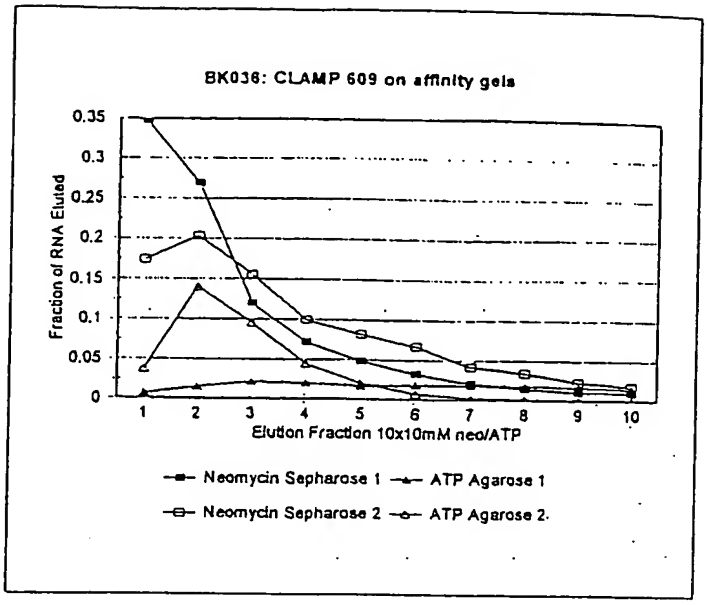
Expt BK036
printed 13-May-02
CLAMP 609

Neomycin Sepharose sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	14712.5	14672	9	132048		129407.0	100%
wash	387.5	347	50	17350	13.14%		0%
wash	40.5	0	50	0	0.00%		
1	9017.5	8977	5	44885	33.99%	44885.0	34.69%
2	7018.5	6978	5	34890	26.42%	34890.0	26.96%
3	3138	3098.5	5	15477.5	11.72%	15477.5	11.96%
4	1895	1854.5	5	9272.5	7.02%	9272.5	7.17%
5	1284.5	1244	5	6220	4.71%	6220.0	4.81%
6	874.5	834	5	4170	3.16%	4170.0	3.22%
7	573.5	533	5	2665	2.02%	2665.0	2.06%
8	413.5	373	5	1865	1.41%	1865.0	1.44%
9	309	268.5	5	1342.5	1.02%	1342.5	1.04%
10	298.5	256	5	1280	0.97%	1280.0	0.99%
BKG		40.5					
% acid insoluble 98.0%					106%	Eluted 122068	94.33%

ATP Agarose sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	21260.5	21220	9	190980		187160.4	100%
wash	1026.5	986	50	49300	25.81%		0%
wash	173.5	133	50	6650	3.48%		
1	289	248.5	5	1242.5	0.65%	1242.5	0.66%
2	589.5	559	5	2795	1.46%	2795	1.49%
3	853.5	813	5	4065	2.13%	4065	2.17%
4	780.5	740	5	3700	1.94%	3700	1.98%
5	667	626.5	5	3132.5	1.64%	3132.5	1.67%
6	728.5	688	5	3440	1.80%	3440	1.84%
7	719	678.5	5	3392.5	1.78%	3392.5	1.81%
8	686	645.5	5	3227.5	1.69%	3227.5	1.72%
9	683	642.5	5	3212.5	1.68%	3212.5	1.72%
10	592.5	552	5	2760	1.45%	2760	1.47%
BKG		40.5					
% acid insoluble 98.0%					46%	Eluted 30968	16.55%

Neomycin Sepharose sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	8283	8283	9	74547		73056.1	100%
wash	67.5	67.5	50	3375	4.53%		
wash	23.5	23.5	50	1175	1.58%		
1	2544.5	2544.5	5	12722.5	17.07%	12722.5	17.41%
2	2970.5	2970.5	5	14852.5	19.92%	14852.5	20.33%
3	2268	2268	5	11340	15.21%	11340.0	15.52%
4	1453	1453	5	7265	9.75%	7265.0	9.94%
5	1196	1196	5	5980	8.02%	5980.0	8.19%
6	977.5	977.5	5	4887.5	6.56%	4887.5	6.69%
7	608	608	5	3040	4.08%	3040.0	4.16%
8	499	499	5	2495	3.35%	2495.0	3.42%
9	334.5	334.5	5	1672.5	2.24%	1672.5	2.29%
10	262.5	262.5	5	1312.5	1.76%	1312.5	1.80%
BKG		23.5					
% acid insoluble 98.0%					94%	Eluted 65568	89.75%

ATP Agarose sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	8184	8127.5	9	73147.5		71684.55	100%
wash	596	539.5	50	26975	36.88%		0%
wash	171.5	115	50	5750	7.86%		
1	587.5	531	5	2655	3.63%	2655	3.70%
2	2057	2000.5	5	10002.5	13.67%	10002.5	13.95%
3	1423	1366.5	5	6832.5	9.34%	6832.5	9.53%
4	686.5	630	5	3150	4.31%	3150	4.39%
5	350.5	294	5	1470	2.01%	1470	2.05%
6	162.5	106	5	530	0.72%	530	0.74%
7	93.5	37	5	185	0.25%	185	0.26%
8	67.5	11	5	55	0.08%	55	0.08%
9	56.5	0	5	0	0.00%	0	0.00%
10	57	0.5	5	2.5	0.00%	2.5	0.00%
BKG		56.5					
% acid insoluble 98.0%					79%	Eluted 24883	34.71%



~~CO~~36

Protocol #: 3 Name: 32PMNH 13:21

S#

CLAMP 609 binding to ATP gel w/"unlocking" by neomycin

CLAMP 609 (~~BKO~~ ^{35}S)

Neomycin Sepharose (new) for control ; ATP agarose (used)

Binding Buffer (Neo): 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6

Binding Buffer (ATP): 1mM neomycin in above

Elution Buffer (Neo) 10mM neomycin in

Elution Buffer (ATP) 10mM ATP in

} old solns ⇒ need to make fresh

~~Wash~~ Wash columns 10x w/100ul Binding Buffer.

Add 4ul ^{RNA} ⇒ 20ul sample. Remove 2ul ⇒ 20ul for total cpm count.

Incubate remaining sample 1 min @ 85°C, cool 1 min, add to columns.

Wash 20x w/100ul Binding Buffer, collecting each 1 ml.

Elute 10x w/100ul Elution Buffer, collecting each 100ul.

4-2F Assay 20ul / fx in 5 ml Scintiverse

* Forced drip-quick flow
* Columns still hot after elution

Samples:	1	Control 20ul/20ul	1/9 sample	} Neomycin sepharose
	2-3	Washes 20ul/1000ul		
	4-13	Elution 20ul/100ul		
	14	Control 20ul/20ul	1/9 sample	} ATP agarose
	15-16	Washes 20ul/1000ul		
	17-26	Elution 20ul/100ul		
	27-28	Column wash 20ul/1000ul		

⇒ still low elution from ATP column

Protocol #: 3 Name: 32PMNH

CLAMP 609 binding to ATP gel w/ "unlocking" by neomycin (repeat)

CLAMP 609 (BKO[redacted])

Neomycin sepharose (neo) for control & ATP agarose (used)

(Neo) Binding Buffer: 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6

(ATP) ~~Bin~~ Binding Buffer: 1mM neomycin in

Elution Buffer (Neo): 10mM neomycin in

Elution Buffer (ATP): 10mM ATP in

Wash column 10x w/100ul Binding Buffer.

Add 2ul RNA → 20ul sample. Remove 2ul → 20ul for total cpm.

Incubate remaining sample 1 min @ 85°C, cool 1 min, add to column.

Wash 20x w/100ul Binding Buffer, collecting each 1 ml.

Elute 10x w/100ul Elution Buffer; collecting each 100ul.

Assay 20ul / fx in 5ml Scintiverse

* Slower drip rate
* Columns only slightly hot after elution (~300 cpm)

Samples:					
1	Control cpm	20ul/20ul	1/9 sample	Neomycin sepharose
2-3	Washes	20ul/1000ul		
4-13	Elution	20ul/100ul		
14	Control cpm	20ul/20ul	1/9 sample	ATP agarose
15-16	Column Prewashes	20ul/1000ul		
17-18	Washes	20ul/1000ul		
19-28	Elution	20ul/100ul		
29-30	Column Washes	20ul/1000ul		

Protocol #: 3 Name:32PMNH 13:21
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 2.00 QIP = SIS

S#	TIME	CPMA	CPMB	B:2S%	SIS	FLAG
1	2.00	14712.5	11394.5	1.32	627.11	
2	2.00	387.50	304.00	8.11	628.76	
3	2.00	40.50	25.50	28.01	381.93	
4	2.00	9017.50	6926.50	1.70	618.68	
5	2.00	7018.50	5178.00	1.97	553.75	
6	2.00	3136.00	2218.00	3.00	449.81	
7	2.00	1895.00	1422.00	3.75	562.68	
8	2.00	1284.50	872.50	4.79	428.33	
9	2.00	874.50	559.00	5.98	405.60	
10	2.00	573.50	438.50	6.77	583.56	
11	2.00	413.50	312.00	8.01	564.44	
12	2.00	309.00	224.50	9.44	522.36	
13	2.00	296.50	202.50	9.94	459.84	
14	2.00	21260.5	17039.0	1.08	679.43	
15	2.00	1026.50	780.50	5.06	581.78	
16	2.00	173.50	114.50	13.22	444.05	
17	2.00	269.00	198.50	10.09	476.83	
18	2.00	598.50	428.00	8.84	544.94	
19	2.00	853.50	621.50	5.67	561.07	
20	2.00	780.50	584.50	5.80	602.36	
21	2.00	667.00	437.00	8.77	422.81	
22	2.00	728.50	528.00	6.15	483.34	
23	2.00	719.00	521.00	6.20	516.21	
24	2.00	686.00	506.50	6.28	557.88	
25	2.00	683.00	562.00	5.97	690.32	
26	2.00	592.50	494.00	8.36	700.70	
27	2.00	623.00	437.00	8.77	535.24	
28	2.00	108.50	81.00	15.71	495.06	

Protocol #: 3 Name:32PMNH 10:21
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 2.00 QIP = SIS

S#	TIME	CPMA	CPMB	B:2S%	SIS	FLAG
1	2.00	8283.00	6509.50	1.75	616.07	
2	2.00	67.50	44.00	21.32	570.14	
3	2.00	23.50	10.50	43.64	239.09	
4	2.00	2544.50	1837.00	3.30	482.79	
5	2.00	2970.50	2130.00	3.06	532.72	
6	2.00	2268.00	1735.50	3.39	584.36	
7	2.00	1453.00	1134.50	4.20	640.96	
8	2.00	1196.00	918.50	4.67	635.13	
9	2.00	977.50	791.50	5.03	711.35	
10	2.00	608.00	403.00	7.04	416.83	
11	2.00	499.00	384.00	7.22	543.59	
12	2.00	334.50	228.00	9.37	429.98	
13	2.00	262.50	185.50	10.38	484.77	
14	2.00	8184.00	8188.50	1.80	555.04	
15	2.00	21.50	9.50	45.88	247.09	
16	2.00	22.50	10.50	43.64	465.13	
17	2.00	596.00	442.50	6.72	538.64	
18	2.00	171.50	122.00	12.80	478.34	
19	2.00	587.50	409.00	6.98	501.26	
20	2.00	2057.00	1580.00	3.56	591.29	
21	2.00	1423.00	1124.00	4.22	635.41	
22	2.00	686.50	565.50	5.95	693.13	
23	2.00	350.50	288.50	8.33	694.04	
24	2.00	162.50	120.00	12.91	507.30	
25	2.00	93.50	67.50	17.21	560.65	
26	2.00	67.50	43.00	21.57	490.62	
27	2.00	56.50	41.50	21.95	501.46	
28	2.00	57.00	38.50	22.79	496.97	
29	2.00	77.50	48.50	20.31	452.02	
30	2.00	39.50	23.50	29.17	291.04	

EXHIBIT C

TITLE: Column Affinity - Neomycin Unlocking of CLAMP 609 on ATP agarose	DATE:
PURPOSE: To determine if neomycin binding can increase ATP binding ability of Neo/ATP-CLAMP 609; repeated three times	EXP.# BK038

REAGENTS

DEPC treated water
^{32}P-Neo-uu-ATP-CLAMP 609 (~ 1.3pmol/ul)/BK037
(gcuuaauacgacucacuauaggccuggggcgagaaguuuaggcc uu
ggguuggggaagaaacuguggcacuucggugccagcaaccc)
ATP Agarose from Sigma No. A2767
MicroBioSpin Column from BioRad No. 732-6204
Neomycin from Sigma No. N-1876 Lot 90K0854 MW 908.9
Adenosine 5'Triphosphate (ATP) from Sigma No. A-3377 MW 551.1
Binding Buffer: 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6
Elution Buffer: 10mM ATP in Binding Buffer
Fisher Scientific ScintiVerse Scintanalyzed Cocktail No. SX18-4 Lot 994498
Packard TR1600 Liquid Scintillation Analyzer (1238 Molec Biol) - Protocol 3

STEP	SOLUTION		VOL (ml)	TEMP (°C)	Time Period
Make Column	Layer 100ul of ATP agarose into micro biospin column.		100ul	23	30sec
Wash Column	Equilibrate column by washing 10x with 100ul Binding Buffer, collecting 1ml.	± 1 mM neomycin	1ml	23	5min
Prepare Samples	~ 3pmol ^{32}P-CLAMP609 Binding buffer	± 1 mM neomycin	20ul	85 23	1min 1min
Apply Sample	Apply sample to column.		18ul	23	30sec
Wash	Wash 20x with 100ul Binding Buffer, collecting each 1ml.	± 1 mM neomycin	2x1ml/fx	23	5min
Elution	Elute 10x with 100ul Elution Buffer, collecting each 1ml.	± 1 mM neomycin	10x100ul/fx	23	10min
Column Washes	Wash column with 1ml Elution Buffer, then with 1ml Binding Buffer, collecting each 1ml.		2x1ml/fx	23	5min
Analyze Fractions	Add 20ul each fraction to 5ml ScintiVerse and assay on liquid scintillator. Reserve 2ul of sample for total sample cpm count.		20ul in 5ml	23	64min

DISCUSSION:

The addition of neomycin to the solutions increased binding to the ATP column from 9% to 28% of TCA precipitable counts in the first trial, from 25% to 59% in the second trial, and from 9% to 25% in the third trial. This suggests that neomycin binding is required in order for the ATP aptamer end of the CLAMP to be fully available for binding. However, the ATP binding is still very low compared to the neomycin binding attained, so other factors probably still reduce this binding, such as incomplete transcription or remaining interference within the CLAMP construct.

printed 09-Sep-02

CLAMP 609 on ATP Agarose

sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	11184	11142.5	9	100282.5		93262.7	100%
wash	1360.5	1339	50	66950	66.8%		0%
wash	199	177.5	50	8875	8.8%		
1	372	350.5	5	1752.5	1.7%	1752.5	1.9%
2	489.5	468	5	2340	2.3%	2340.0	2.5%
3	349	327.5	5	1637.5	1.6%	1637.5	1.8%
4	260.5	239	5	1195	1.2%	1195.0	1.3%
5	120	98.5	5	492.5	0.5%	492.5	0.5%
6	72.5	51	5	255	0.3%	255.0	0.3%
7	53.5	32	5	160	0.2%	160.0	0.2%
8	37	15.5	5	77.5	0.1%	77.5	0.1%
9	39	17.5	5	87.5	0.1%	87.5	0.1%
10	36.5	15	5	75	0.1%	75.0	0.1%
BKG		21.5				Eluted	
% acid insoluble		93.0%			83.68%	8073	9%

CLAMP 609 on ATP Agarose - with neomycin unlocking

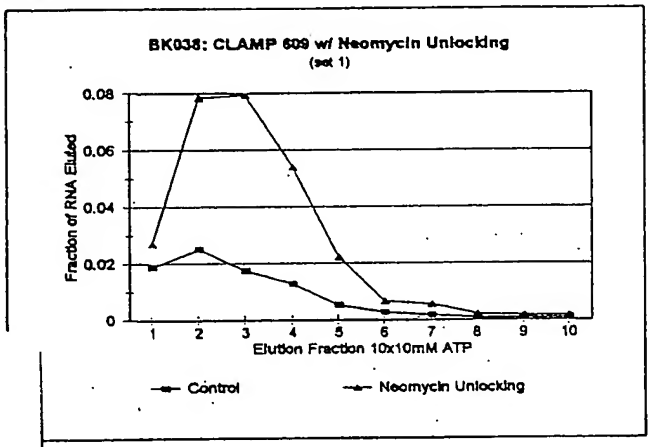
sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	11832	11811.5	9	106303.5		98862.26	100%
wash	1274.5	1254	50	62700	59%		0%
wash	249	228.5	50	11425	10.75%		
1	556	534.5	5	2672.5	2.51%	2672.5	2.70%
2	1571.5	1551	5	7755	7.30%	7755	7.84%
3	1590	1569.5	5	7847.5	7.38%	7847.5	7.94%
4	1090	1069.5	5	5347.5	5.03%	5347.5	5.41%
5	462	441.5	5	2207.5	2.08%	2207.5	2.23%
6	152	131.5	5	657.5	0.62%	657.5	0.67%
7	131.5	111	5	555	0.52%	555	0.56%
8	64	43.5	5	217.5	0.20%	217.5	0.22%
9	55.5	35	5	175	0.16%	175	0.18%
10	55	34.5	5	172.5	0.16%	172.5	0.17%
BKG		20.5				Eluted	
% acid insoluble		93.0%			95.70%	27608	28%

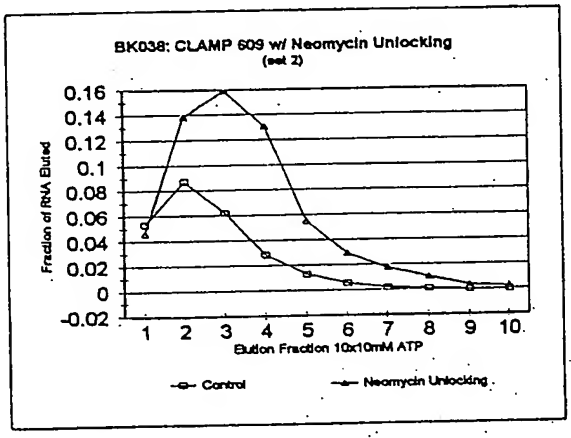
CLAMP 609 on ATP Agarose

sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	5477	5444.5	9	49000.5		45570.47	100%
wash	1013	980.5	50	49025	100%		0%
wash	187	154.5	50	7725	15.77%		
1	520.5	488	5	2440	4.98%	2440	5.35%
2	829	798.5	5	3982.5	8.13%	3982.5	8.74%
3	602	569.5	5	2847.5	5.81%	2847.5	6.25%
4	294.5	262	5	1310	2.67%	1310	2.87%
5	152.5	120	5	600	1.22%	600	1.32%
6	88	53.5	5	267.5	0.55%	267.5	0.59%
7	51.5	19	5	95	0.19%	95	0.21%
8	40	7.5	5	37.5	0.08%	37.5	0.08%
9	30.5	-2	5	-10	-0.02%	-10	-0.02%
10	30	-2.5	5	-12.5	-0.03%	-12.5	-0.03%
BKG		32.5				Eluted	
% acid insoluble		93.0%			139.40%	11558	25%

CLAMP 609 on ATP Agarose - with neomycin unlocking

sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	4437.5	4401	9	39609		36836.37	100%
wash	859.5	823	50	41150	104%		0%
wash	197.5	161	50	8050	20.32%		
1	379.5	343	5	1715	4.33%	1715	4.66%
2	1059	1022.5	5	5112.5	12.91%	5112.5	13.88%
3	1211	1174.5	5	5872.5	14.83%	5872.5	15.94%
4	1002.5	968	5	4830	12.19%	4830	13.11%
5	448.5	410	5	2050	5.18%	2050	5.57%
6	254.5	218	5	1090	2.75%	1090	2.96%
7	168.5	130	5	650	1.64%	650	1.76%
8	113.5	77	5	385	0.97%	385	1.05%
9	62.5	26	5	130	0.33%	130	0.35%
10	53	16.5	5	82.5	0.21%	82.5	0.22%
BKG		36.5				Eluted	
% acid insoluble		93.0%			179.55%	21918	59%



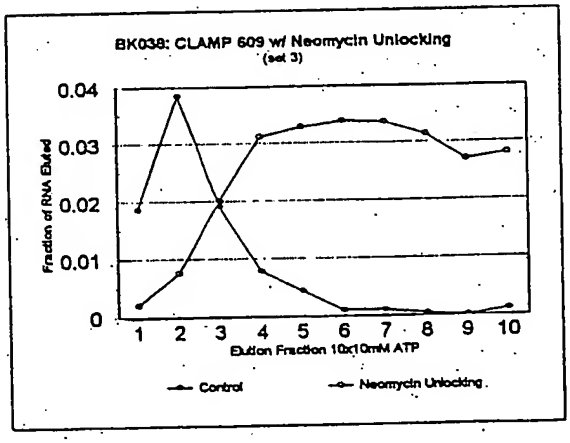


CLAMP 609 on ATP Agarose

sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	8881	8856	9	79704		74124.72	100%
wash	1114	1089	50	54450	68%		0%
wash	218.5	193.5	50	9675	12.14%		
1	303	278	5	1390	1.74%	1390	1.88%
2	596	571	5	2855	3.58%	2855	3.85%
3	311	286	5	1430	1.79%	1430	1.93%
4	142.5	117.5	5	587.5	0.74%	587.5	0.79%
5	91	66	5	330	0.41%	330	0.45%
6	40.5	15.5	5	77.5	0.10%	77.5	0.10%
7	41	16	5	80	0.10%	80	0.11%
8	32	7	5	35	0.04%	35	0.05%
9	25.5	0.5	5	2.5	0.00%	2.5	0.00%
10	42	17	5	85	0.11%	85	0.11%
BKG		25				Eluted	
% acid insoluble		93.0%			89.08%	6873	9%

CLAMP 609 on ATP Agarose - with neomycin unlocking

sample	value	value - bkg	dilution factor	total cpm	%input	Acid precipitable cpm	% total
input	1554	1507	9	13563		12613.59	100%
wash	114.5	67.5	50	3375	25%		0%
wash	26.5	-20.5	50	-1025	-7.56%		
1	52.5	5.5	5	27.5	0.20%	27.5	0.22%
2	66.5	19.5	5	97.5	0.72%	97.5	0.77%
3	98	51	5	255	1.88%	255	2.02%
4	128	79	5	395	2.91%	395	3.13%
5	130	83	5	415	3.06%	415	3.29%
6	132.5	85.5	5	427.5	3.15%	427.5	3.39%
7	132	85	5	425	3.13%	425	3.37%
8	126.5	79.5	5	397.5	2.93%	397.5	3.15%
9	115.5	68.5	5	342.5	2.53%	342.5	2.72%
10	118	71	5	355	2.62%	355	2.81%
BKG		47				Eluted	
% acid insoluble		93.0%			40.46%	3138	25%



"Unlocking" effect of neomycin on CLAMP609 ATP binding

CLAMP609 (BK0[redacted]37

ATP agarose (used)

Binding Buffer (control): 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH7.6

Binding Buffer (unlocking): 1mM neomycin, 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH7.6

Elution Buffer: 10mM ATP, 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6

Elution Buffer (unlocking): above plus 1mM neomycin

~~Wash column 10x w/100ul Elution Buffer, collecting 1ml.~~

Wash column 10x w/100ul Binding Buffer, collecting 1 ml.

Add 2ul RNA → 20ul sample (B.B. dilutes). Remove 2ul → 20ul for total cpm.

Incubate remaining sample 1 min @ 85°C, cool 1 min, add to column.

Wash 20x w/100ul Binding Buffer, collecting each 1 ml.

Elute 10x w/100ul Elution Buffer, collecting each 100 ul.

Wash column 10x w/100ul Elution Buffer, collecting 1 ml.

Wash column 10x w/100ul Binding Buffer w/o neomycin, collecting 1 ml.

Assay 20ul / fx in 5ml Scintiverse.

* columns only slightly lit afterwards

Samples:						
1	Control	20ul/20ul 1/9 sample		17		
2	Column Re-Wash	20ul/1000ul		18		
3-4	Washes	20ul/1000ul	Control	19-20	Unlocking	
5-14	Elution	20ul/100ul	9%	21-30	28%	
15-16	Column Washes	20ul/1000ul		31-32		

Repeat exactly as above except neither elution buffer contains neomycin

Control - 25% Unlocking 59%

Repeat as above w/ elution buffer w/o neomycin

Control 9% Unlocking 25%

Protocol #: 3 Name:32PMNH 14:20
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
e = 2.00 QIP = SIS

S#	TIME	CPMA	CPMB	B:2S%	SIS	FLAG
1	2.00	11164.0	8355.50	1.55	544.96	
2	2.00	21.50	10.50	43.64	451.41	
3	2.00	1360.50	1064.50	4.33	641.54	
4	2.00	199.00	129.50	12.43	398.68	
5	2.00	372.00	258.00	8.80	529.43	
6	2.00	489.50	372.50	7.33	557.94	
7	2.00	349.00	242.00	9.09	510.14	
8	2.00	260.50	178.50	10.59	518.90	
9	2.00	120.00	82.00	15.62	432.42	
10	2.00	72.50	53.50	19.33	641.54	
11	2.00	53.50	30.50	25.61	427.76	
12	2.00	37.00	22.50	29.81	473.56	
13	2.00	39.00	19.00	32.44	273.50	
14	2.00	36.50	16.00	35.36	436.39	
15	2.00	26.50	12.50	40.00	334.99	
16	2.00	36.00	22.50	29.81	415.56	
17	2.00	11832.0	8961.00	1.49	599.53	
18	2.00	20.50	8.50	48.51	124.50	
19	2.00	1274.50	951.50	4.58	523.71	
20	2.00	249.00	163.50	11.06	480.42	
21	2.00	555.00	406.00	7.02	543.77	
22	2.00	1571.50	1116.50	4.23	469.11	
23	2.00	1590.00	1200.00	4.08	566.86	
24	2.00	1090.00	774.50	5.08	503.81	
25	2.00	462.00	326.00	7.83	512.29	
26	2.00	152.00	95.50	14.47	386.64	
27	2.00	131.50	95.50	14.47	521.19	
28	2.00	64.00	35.50	23.74	397.77	
29	2.00	55.50	38.50	22.79	535.36	
30	2.00	55.00	30.00	25.82	446.85	
31	2.00	37.00	19.00	32.44	497.00	
32	2.00	29.00	15.50	35.92	565.03	

32(2)

Protocol #: 3 Name:32PMNH 16:50
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 2.00 QIP = SIS

S#	TIME	CPMA	CPMB	B:2S%	SIS	FLAG
1	2.00	5477.00	4327.00	2.15	630.49	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
6	2.00	829.00	624.00	5.66	553.51	
7	2.00	602.00	470.50	6.52	631.53	
8	2.00	294.50	221.00	9.51	539.05	
9	2.00	152.50	112.00	13.36	572.09	
10	2.00	86.00	60.50	18.18	452.53	
11	2.00	51.50	31.50	25.20	464.95	
12	2.00	40.00	22.00	30.15	315.86	
13	2.00	30.50	15.00	36.51	318.22	
14	2.00	30.00	13.50	38.49	341.19	
15	2.00	24.00	14.00	37.80	378.88	
16	2.00	42.00	24.50	28.57	312.21	
17	2.00	4437.50	3492.50	2.39	601.46	
18	2.00	36.50	24.00	28.87	460.09	
19	2.00	859.50	607.50	5.74	513.46	
20	2.00	197.50	150.50	11.53	569.37	
21	2.00	379.50	271.50	8.58	507.38	
22	2.00	1059.00	782.50	5.06	534.56	
23	2.00	1211.00	908.00	4.69	557.88	
24	2.00	1002.50	705.00	5.33	524.97	
25	2.00	446.50	330.50	7.78	501.70	
26	2.00	254.50	180.50	10.53	542.42	
27	2.00	166.50	120.50	12.88	492.37	
28	2.00	113.50	79.00	15.91	501.40	
29	2.00	62.50	40.00	22.36	448.20	
30	2.00	53.00	35.00	23.90	487.85	
31	2.00	34.00	18.50	32.88	398.72	
32	2.00	66.50	41.50	21.95	491.40	

30
(3)

Protocol #: 3 Name:32PMNH 09:44
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.10
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 2.00 QIP = SIS

S#	TIME	CPMA	CPMB	B:2S%	SIS	FLAG
1	2.00	8881.00	7140.00	1.67	685.52	
2	2.00	25.00	10.00	44.72	424.10	
3	2.00	1114.00	818.50	4.94	542.31	
4	2.00	218.50	163.00	11.08	529.52	
5	2.00	303.00	243.50	8.06	625.26	
6	2.00	596.00	472.50	6.51	675.09	
7	2.00	311.00	227.00	9.38	551.88	
8	2.00	142.50	95.00	14.51	443.71	
9	2.00	91.00	62.00	17.96	440.44	
10	2.00	40.50	23.50	29.17	345.52	
11	2.00	41.00	21.50	30.50	332.35	
12	2.00	32.00	19.50	32.03	360.78	
13	2.00	25.50	12.00	40.82	552.16	
14	2.00	42.00	22.50	29.81	351.20	
15	2.00	28.50	13.00	39.22	532.13	
16	2.00	39.50	20.50	31.23	425.34	
17	2.00	1554.00	1151.00	4.17	559.42	
18	2.00	47.00	31.50	25.20	165.14	
19	2.00	114.50	85.50	15.29	537.42	
20	2.00	26.50	16.50	34.82	535.61	
21	2.00	52.50	31.00	25.40	529.57	
22	2.00	66.50	47.00	20.63	514.24	
23	2.00	98.00	71.00	16.78	565.60	
24	2.00	126.00	83.00	15.52	490.09	
25	2.00	130.00	90.50	14.87	572.18	
26	2.00	132.50	84.00	15.43	448.77	
27	2.00	132.00	83.50	15.48	407.11	
28	2.00	126.50	75.00	16.33	420.04	
29	2.00	115.50	78.50	15.86	521.63	
30	2.00	118.00	80.00	15.81	553.93	
31	2.00	76.00	53.50	19.33	597.11	
32	2.00	68.00	44.00	21.32	510.19	

EXHIBIT D

TITLE: Native PAGE - Neomycin Effects on CLAMP Binding to Affinity Gels	DATE: [illegible]
PURPOSE: To test the effects of different neomycin concentrations on CLAMP 609 binding to affinity gels	EXP.# BK041

REAGENTS

^{32}P-Neo-uu-ATP-CLAMP 609 (~ 2pmol/ul)/ BK040
(gcuuaauacgacucacuauaggccuggggcgagaaguuuaggcc uu
ggguuggggaagaaacuguggcacuucggugccagcaaccc)
Neomycin Sepharose from BK001
ATP Agarose from Sigma No. A2767
Neomycin from Sigma No. N-1876 Lot 90K0854 MW 908.9
Binding Buffer: 50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6
Neomycin Solution: 10mM neomycin in Binding Buffer
Nondenaturing gel 5x loading dye (bromophenol blue and xylene cyanol FF dyes)
Nondenaturing 12% acrylamide gel, TBE Buffer, PAGE apparatus, Saran Wrap
Whatman Chromatography Paper No. 3003917, Gel Drying Film from Promega No. V7131
BioRad Model 583 Gel Dryer, Molecular Dynamics Storage Phosphor Screen & Cassette
Molecular Dynamics Phosphorlmager Model 400A (2127 MBB)

STEP	SOLUTION			VOL (ml)	TEMP (°C)	Time Period
Prepare Samples	~ 4pmol ^{32}P-Neo/ATP-CLAMP 50mM Tris 250mM NaCl 5mM $MgCl_2$ pH 7.6	± neomycin (0.1, 0.5, 1, 10 mM)	±neomycin-sepharose ± ATP-agarose	8 ul	23	10min
Load Gel	Add loading dye to above and load samples on gel, skipping a well after sample 1 to mark lane 1.			10ul	23	5min
Run Gel	Run native PAGE electrophoresis at 30mA/gel			1 gel	23	1.5hr
Dry Gel	Dry gel between layers of gel drying film on Whatman paper.			1 gel	80	1hr
Expose to Screen	Wrap dried gel in plastic wrap and place in phosphoimager cassette ovemight.			1 gel	23	18hr
Image	Record image on phosphoimager. (Gel# 17010)					

DISCUSSION:
The image of this gel had background spots and the bands were less even, but affinity gel bound CLAMP was still visible retained in the wells. More CLAMP was visible in the free bands in the gel, possibly due to the lower ligand concentration of the affinity gels as compared to the derivatized microspheres. The most interesting results from this gel is seen in samples 7-11, where as neomycin concentration is increased, the bands of free CLAMP decrease in intensity, suggesting that ATP agarose binding increases. This reinforces the results from column affinity studies (BK036 & BK038) that showed the presence of free neomycin increasing the adenosine binding capacity of CLAMP 609.

C:\DATA\BK041B.GEL , Range = 0.01-900.00 Counts, 1.00x

Native PAGE – CLAMP609 binding to affinity gels (BK040)

BK041

12% acrylamide gel

30% acrylamide	8.0 ml
DEPC water	9.86 ml
10x TBE	2.0 ml
10% ammonium persulfate	0.14 ml
TEMED	7 ul

Binding Buffer: 50mM Tris, 250mM NaCl, 5mM MgCl, pH 7.6

Torrey's Nondenaturing Gel 5x Loading Dye w/ bromophenol blue & xylene cyanol FF
70bp 20bp

cc #17010

Sample#	CLAMP 609	5x dye	Neo. Soph.	Aden. Agm.	B.B.	B.B. w/ 50mM Neo.	B.B. w/ 2.5mM neo	final [neomycin]
1	2 ul	2 ul	—	—	6	—	—	0 mM
2	2	2	2	–	4	—	—	0
3	2	2	2	–	3.6	[illegible]	0.4	0.1
4	2	2	2	–	[illegible]	[illegible]	2	0.5
5	2	2	2	–	3.8	0.2	—	1
6	2	2	2	–	2	2	—	10
7	2	2	–	2	4	—	—	0
8	2	2	–	2	3.6	[illegible]	0.4	0.1
9	2	2	–	2	[illegible]	[illegible]	2	0.5
10	2	2	–	2	3.8	0.2	—	1
11	2	2	–	2	2	2	—	10
12	2	2	2	2	2	—	—	0
13	2	2	2	2	1.6	[illegible]	0.4	0.1
14	2	2	2	2	[illegible]	[illegible]	2	0.5
15	2	2	2	2	1.8	0.2	—	1
16	2	2	2	2	—	2	—	10

Allow gel to polymerize 1hr. Run gel @ 30mA for 1.5 hr. Dry w/ plastic membrane 1hr @ 80°C.
Expose to phosphorimager screen overnight (18hr).

⇒ overloaded, spotty gel

:\DATA\BK041B.GEL , Range = 0.01-900.00 Counts, 1.00x

Gel #17010